Research Article

Endogenous Cytosine Damage Products Alter the Site Selectivity of Human DNA Maintenance Methyltransferase DNMT1

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Abstract

Alterations in cytosine methylation patterns are usually observed in human tumors. The consequences of altered cytosine methylation patterns include both inappropriate activation of transforming genes and silencing of tumor suppressor genes. Despite the biological effect of methylation changes, little is known about how such changes are caused. The heritability of cytosine methylation patterns from parent to progeny cells is attributed to the fidelity of the methylation-sensitive human maintenance methyltransferase DNMT1, which methylates with high specificity the unmethylated strand of a hemimethylated CpG sequence following DNA replication. We have been studying DNA damage that might alter the specificity of DNMT1, either inhibiting the methylation of hemimethylated sites or triggering the inappropriate methylation of previously unmethylated sites. Here, we show that known forms of endogenous DNA damage can cause either hypermethylation or hypomethylation. Inflammation-induced 5-halogenated cytosine damage products, including 5-chlorocytosine, mimic 5-methylcytosine and induce inappropriate DNMT1 methylation within a CpG sequence. In contrast, oxidation damage of the methyl group of 5-methylcytosine, with the formation of 5-hydroxymethylcytosine, prevents DNMT1 methylation of the target cytosine. We propose that reduced DNMT1 selectivity resulting from DNA damage could cause heritable changes in cytosine methylation patterns, resulting in human tumor formation. These data may provide a mechanistic link for the associations documented between inflammation and cancer. [Cancer Res 2007;67(3):946–50]

Introduction

Alterations in cytosine methylation patterns observed in human tumors include both local hypermethylation accompanied by gene silencing and global hypomethylation. The list of genes whose expression is altered by changes in cytosine methylation in human tumors is growing rapidly (1–5). However, the mechanisms by which these epigenetic alterations arise in the development of cancer are still unknown.

The epigenetic code in human cells involves both changes in cytosine methylation patterns as well as covalent modifications to histone proteins (6, 7). It is currently unknown if cytosine methylation leads to histone modification or if histone modification induces DNA methylation changes or both. It is known that human cells contain a family of proteins that bind with high affinity to DNA sequences containing the CpG dinucleotide when these sequences contain 5-methylcytosine in both strands (8). These methyl-binding proteins (MBP) also have affinity for histone-modifying enzymes. It is currently thought that the initial binding of MBPs to methylated CpG sequences triggers a cascade of events via the recruitment of histone-modifying enzymes, leading to chromatin condensation and gene silencing (9).

Chemical damage to DNA could conceivably alter epigenetic patterning by interfering with the initial binding of the MBPs. We have previously shown that oxidation of a guanine residue to 8-oxoguanine or the oxidation of 5-methylcytosine to 5-hydroxymethylcytosine within a CpG target sequence reverses the affinity of the MBPs resulting from cytosine methylation (10). In contrast, the presence of 5-chlorocytosine or 5-bromocytosine within the CpG dinucleotide mimics cytosine methylation (11). The MBPs do not distinguish chlorinated or brominated DNA from methylated DNA. Although 5-halopyrimidines have been used in previous studies of DNA-protein interactions (12, 13), it has only been recognized recently that 5-chlorocytosine and 5-bromocytosine are formed *in vivo* by inflammation-mediated reactive molecules (14–17). Furthermore, 5-chlorocytosine has only recently been made available by synthetic methods developed in this laboratory, allowing studies with the complete 5-halogenated cytosine series (18).

DNA damage-mediated alteration in MBP binding could conceivably influence the recruitment of histone-modifying enzymes, ultimately leading to changes in epigenetic patterns. However, a more direct mechanism by which DNA damage could lead to heritable changes in cytosine methylation patterns is by direct interference with the site-specific methylation of DNA by the maintenance methyltransferase DNMT1. Once cytosine methylation patterns have been established in mammalian cells, the methylation patterns are heritably transmitted to progeny cells. The mechanistic basis for the heritable transfer of cytosine methylation patterns resides in the specificity of DNMT1. Symmetrically methylated CpG dinucleotides generate hemimethylated sites following DNA replication (5, 6). These hemimethylated sites are the preferred substrate for DNMT1, which regenerates the symmetrically methylated sites (Fig. 1*A*).

Damage to the CpG dinucleotides could interfere with the site-specific methylation of hemimethylated CpG dinucleotides by DNMT1. We have therefore constructed a series of oligonucleotides containing a series of modified cytosine residues and have examined the capacity of these modified cytosine residues to influence the DNA methylation specificity of DNMT1. The modified cytosine residues in this study include those carrying a halogen in the 5-position as well as oxidation damage products of cytosine (5-hydroxycytosine) and 5-methylcytosine (5-hydroxymethylcytosine) and a cytosine containing a bulky alkyl chain, 5-(1-propynyl)-cytosine (pdC; Fig. 2). Our results show that 5-halogenated cytosine damage products, including 5-chlorocytosine, mimic 5-methylcytosine, inducing inappropriate DNMT1 methylation within a CpG sequence, whereas oxidation damage of the methyl group of

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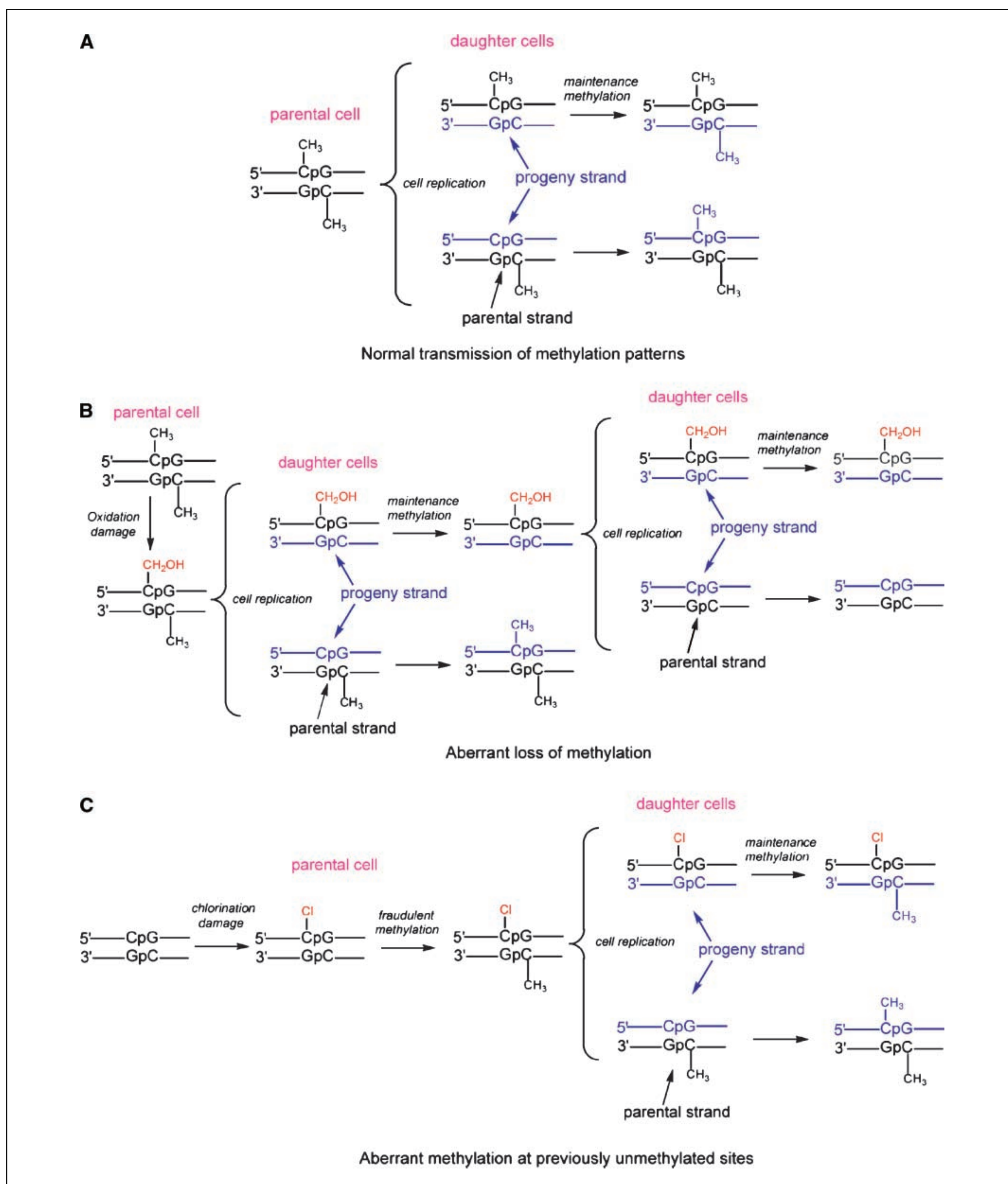

Figure 1. Proposed mechanisms for alterations of maintenance methylation due to DNA damage. *A,* postreplicative maintenance methylation is important in maintaining the faithful heritability of cytosine methylation patterns from parent to progeny. The methyl group of 5-methylcytosine on the parental strand is critical for directing maintenance methylation of the initially unmodified daughter strand in the newly replicated duplex. *B,* oxidation of 5-methylcytosine to 5-hydroxymethylcytosine prevents DNMT1 methylation of cytosine in the opposite strand. Loss of DNMT1 methylation activity on the daughter strand at the oxidized CpG site would result in loss of methylation at that CpG site in the daughter cell. *C,* inflammation-mediated damage products 5-chlorocytosine and 5-bromocytosine direct DNMT1 methylation. Chlorination or bromination of cytosine in a CpG can potentially lead to fraudulent methylation by DNMT1. DNMT1 methylation of the newly replicated daughter strand as directed by DNA damage products can potentially lead to the establishment of aberrant methylation patterns.

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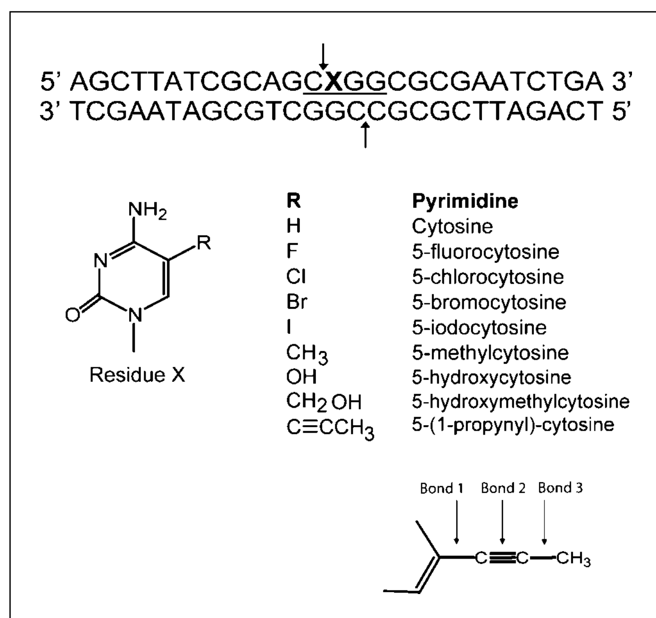

Figure 2. Sequence of 27-mer oligonucleotide duplex used in probing the site selectivity of DNMT1 methyltransferase. The X position indicates the pyrimidine analogue within the central CG of the CCGG recognition sequence of *Hpa*II restriction endonuclease. *Arrows,* cleavage sites for *Hpa*II endonuclease. The structure of the cytosine analogue is shown, where R indicates substitution at the 5-position.

5-methylcytosine prevents DNMT1 methylation of the target cytosine. We propose that reduced DNMT1 selectivity resulting from DNA damage could, in part, cause the aberrant cytosine methylation patterns often observed in human tumors.

Materials and Methods

Oligonucleotide synthesis and purification. Oligonucleotide 27-mers (Fig. 2) containing a central CpG dinucleotide containing modified cytosines (Fig. 2, *top strand*) were prepared by standard solid-phase synthesis using either the Gene Assembler Plus (Pharmacia) or the Expedite Nucleic Acid Synthesis System (Applied Biosystems) automated DNA synthesizers. The oligonucleotide containing pdC at residue X (Fig. 2) was obtained from TriLink Biotechnologies. The duplex sequence used in this study (Fig. 2) was chosen based on binding experiments previously conducted with the MBD of MeCP2 (10). Residue X indicates the positions where cytosine derivatives were systematically substituted. R indicates substituent at the 5-position of residue X that include H, F, Cl, Br, I, CH_3, OH, CH_2OH, and 1-propynyl to make cytosine, 5-fluorocytosine, 5-chlorocytosine, 5-bromocytosine, 5-iodocytosine, 5-methylcytosine, 5-hydroxycytosine, 5-hydroxymethylcytosine, and pdC, respectively. The 5-chlorocytosine, 5-hydroxycytosine, and 5-hydroxymethylcytosine phosphoramidites were prepared according to the methods developed by this laboratory (18, 19). All other phosphoramidites used were obtained from Glen Research. Oligonucleotides were removed from the solid support and deprotected in aqueous ammonia (Aldrich) at 60°C overnight. The deprotected oligonucleotides were purified with Poly-Pak II cartridges (Glen Research). The sequence composition of the oligonucleotides was confirmed via high-performance liquid chromatography analysis following digest of the oligonucleotides with nuclease P1 (Sigma) at 37°C for 1 h and bacterial alkaline phosphatase (Sigma) at 37°C overnight.

DMNT1 methylation protection assay. DNMT1 methyltransferase and appropriate reaction buffers and cofactors were obtained from New England Biolabs. The 27-mer oligonucleotide containing cytosine within the central CpG (Fig. 2, *bottom strand*) was 5′-^{32}P-end labeled by T4 polynucleotide kinase (New England Biolabs) with [γ-^{32}P]ATP (MP Biomedicals) under conditions recommended by the enzyme supplier and subsequently purified using G50 Sephadex columns (Roche). Labeled strands were mixed with 2-fold excess of the complementary strand containing the specific cytosine modifications (Fig. 2, *top strand*) in 10 mmol/L Tris-HCl (pH 7.0) incubated at 95°C for 5 min and allowed to cool to room temperature slowly for duplex formation. The labeled duplex (10 pmol) was incubated with 12 units of DNMT1 (~8 pmol), 1.6 mmol/L S-adenosylmethionine, and 1 μg/mL bovine serum albumin in 1× DNMT1 reaction buffer [50 mmol/L Tris-HCl, 1 mmol/L EDTA, 1 mmol/L DTT, 5% glycerol (pH 7.8)] in a final volume of 25 μL for 4 h at 37°C. One unit of DNMT1 is defined as the amount of enzyme required to catalyze the transfer of 1 pmol of methyl group to poly(deoxyinosinic-deoxycytidylic acid) substrate in a total reaction volume of 25 μL in 30 min at 37°C. The reactions were stopped by incubating at 65°C for 20 min. The stopped reactions were then purified through Sephadex G50 columns and reannealed in 10 mmol/L Tris-HCl with 250-fold excess of the unmodified oligonucleotide with the same sequence as the unlabeled complementary strand that contained the 5-substituted analogues. After reannealing, the duplex (5 pmol) was incubated with 20 units of *Hpa*II restriction endonuclease (New England Biolabs) in a total volume of 25 μL with 1× NEB 1 [10 mmol/L Bis-Tris propane-HCl, 10 mmol/L $MgCl_2$, 1 mmol/L DTT (pH 7.0); New England Biolabs]. One unit of *Hpa*II is defined as the amount of enzyme required to digest 1 μg of λ DNA in 1 h at 37°C in a total reaction volume of 50 μL. The reactions were incubated for 2 h at 37°C and stopped using equal volumes of Maxam-Gilbert loading buffer (98% formamide, 0.01 mol/L EDTA, 1 mg/mL xylene cyanol, 1 mg/mL bromophenol blue). The reaction products were electrophoresed on 20% (v/v) denaturing polyacrylamide gels and visualized and quantified using a phosphorimager

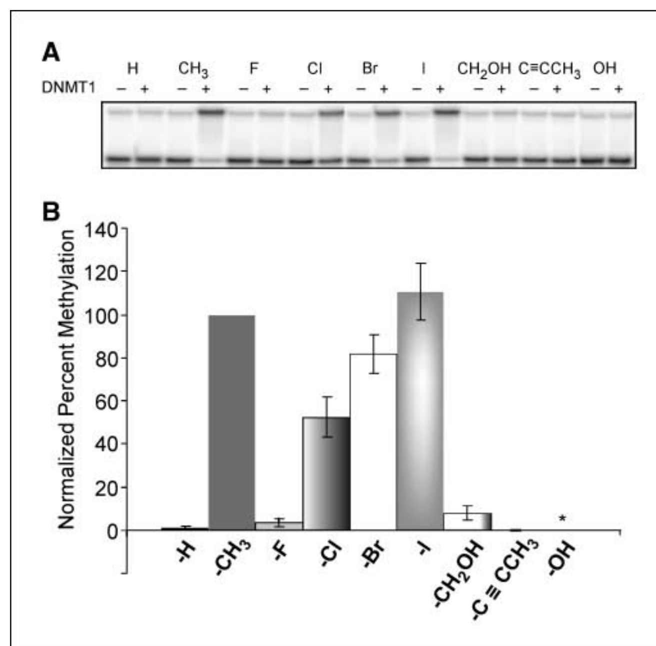

Figure 3. Alteration of site-specific DNMT1 activity by endogenous cytosine damage products. *A,* DNMT1 methylation direction properties of cytosine analogues. *Top band,* oligonucleotide was methylated by DNMT1 and protected from *Hpa*II cleavage; *bottom band,* oligonucleotide was not methylated by DNMT1 and therefore susceptible to *Hpa*II cleavage. Inflammation damage products 5-chorocytosine and 5-bromocytosine within a CpG sequence direct DNMT1 methylation of cytosine in the opposing strand. Oxidative damage products 5-hydroxycytosine and 5-hydroxymethylcytosine do not direct DNMT1 methylation of cytosine in the opposing strand. *B,* DMNT1 percentage methylation of target cytosine opposite various cytosine analogues compared with 5-methylcytosine at 4 h. *Bars,* SD. *Asterisk,* no activity was detected. DNMT1 activity is progressively enhanced with increasing size of the 5-substituent of cytosine on the directing strand until a size limit between 4.23 and 6.1 Å is reached. Although the size of the 5-substituent in hydroxycytosine is below the threshold for DNMT1, it likely disrupts duplex stability, making it unfavorable for DNMT1 recognition.

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Table 1. Size of the 5-substituted cytosine analogues

C-5 substituent	Normalized percentage direction of methylation	van der Waals radius (Å) of C-5 terminal moiety	Bond 1 (Å)	Bond 2 (Å)	Bond 3 (Å)	Size (Å)
H	0.8 ± 0.7	1.2	1.06	—	—	2.26
F	3.5 ± 2.0	1.35	1.30	—	—	2.65
Cl	52 ± 9.5	1.80	1.72	—	—	3.52
Br	82 ± 8.9	1.95	1.87	—	—	3.82
CH_3	100	2.00	1.5	—	—	3.5
I	110 ± 13	2.15	2.08	—	—	4.23
OH	No activity detected	1.2	1.39	0.99	—	3.58
CH_2OH	7.8 ± 3.2	1.2	1.5	1.43	0.99	5.12
$CCCH_3$	0.03 ± 0.03	2.00	1.43	1.2	1.47	6.1

NOTE: The size of the substituent would be the sum of the van der Waals radius and the bond lengths (33). Refer to Fig. 2 for location of bond. Methylation activity of DNMT1 in the presence of the cytosine analogues is reported as percentages normalized to the methylation activity of DNMT1 in the presence of 5-methylcytosine (100%). The average values are reported along with the SD.

(Molecular Dynamics) and the ImageQuant 5.0 software (Molecular Dynamics). The experiments were conducted in triplicate. The data were normalized to the percentage direction of methylation obtained for 5-methylcytosine in each experiment due to variability in activity between lots of the purified DNMT1 obtained.

Results and Discussion

The assay described here to monitor the effect of cytosine analogues on DNMT1 methylation exploits the methylation sensitivity of the *Hpa*II restriction endonuclease. In this assay, a DNA duplex containing a CCGG site is prepared in which a modified cytosine residue is incorporated into one strand and a normal cytosine methylation target residue is contained in a complementary 5′-^{32}P-labeled strand (Fig. 2). Following incubation with purified DNMT1, the test duplex is thermally denatured in the presence of an excess of unlabeled complementary strand containing only normal DNA bases. The duplex formed between the labeled target strand and the unmodified complement is then probed with *Hpa*II. If DNMT1 succeeded in methylating the target strand, it would be protected from subsequent *Hpa*II cleavage (20).

The results of our studies with the cytosine analogues are shown in Fig. 3 and Table 1. In accord with published studies, our results show that DNMT1 has over a 50-fold preference for hemimethylated DNA (5, 6, 21). Oxidation of the hydrophobic methyl group to the hydrophilic hydroxymethyl group interferes with DNMT1-mediated methylation, reducing the modification of the target cytosine residue by >90%. It has been shown previously that the methyl group of 5-methylcytosine is more reactive than the methyl group of thymine and that the production of 5-hydroxymethylcytosine due to hydroxyl radical attack of 5-methylcytosine in duplex DNA *in vivo* is on the order of 40 per cell per day (11, 22).

Another form of oxidative damage to the CpG dinucleotide is oxidation of cytosine forming 5-hydroxycytosine. The presence of the 5-substituent of 5-hydroxycytosine could potentially mimic the methyl group of 5-methylcytosine in directing methylation to the progeny strand. However, we observe that 5-hydroxycytosine is ineffective in directing methylation and, in fact, creates a worse substrate than the unmethylated duplex (Fig. 3). It has been previously reported that 5-hydroxycytosine is one of the most mutagenic of all known DNA damage products (23). Ionization of the 5-hydroxy group under physiologic conditions can trigger a tautomeric shift, allowing the formation of an illegitimate base pair with adenine during DNA replication (24). Transition mutations at CpG dinucleotides represent the most frequent single base change observed in human tumors (25). The formation of 5-hydroxycytosine within the CpG dinucleotide likely contributes to this mutational burden; however, alterations in methylation due to direct stimulation of DNMT1 activity by 5-hydroxycytosine are unlikely.

Previously, it was shown that the oxidation of guanine to 8-oxoguanine within a CpG dinucleotide similarly inhibited DNMT1-mediated methylation (26). In conjunction with the results reported here, it is clear that oxidative damage to DNA generally inhibits DNMT1-mediated methylation and would therefore tend to lead to a loss of methylation in progeny cells (Fig. 1*B*). The magnitude of these changes would increase with increased oxidative stress.

In contrast to oxidation, we show here that 5-substituted cytosine residues containing larger halogens can mimic cytosine methylation and direct DNMT1 to methylate the cDNA strand, in accord with a previous study (13). Although the inductive electronic effect of the halogen substituents is opposite that of the methyl group, the bromo substituent is similar in size to a methyl group. The similarity in the effect of the 5-bromo and 5-methyl substituents on the binding of MBPs as well as on the methylation-directing capacity of DNMT1 suggests that both the MBPs and DNMT1 have a pocket that sterically accommodates the methyl group and that the steric effect dominates the interaction.

To probe the size of this pocket with DNMT1, we examined the entire series of halogen substituents. The smaller fluoro substituent has only a very modest effect on DNMT1-mediated methylation, whereas the larger chloro substituent can stimulate methylation by DNMT1, although the effect is approximately half that of the methyl group. The larger bromo substituent is better than chloro, and the largest halogen substituent, iodo, is the best of the halogens and is even better than methyl. This result suggests that the steric pocket on DNMT1 is larger than the

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methyl group. We therefore used a three-carbon substituent that would project a methyl group further into the pocket. We observe that the pdC analogue is ineffective in stimulating methylation, indicating that the edges of the methyl-binding pocket are encountered between 4.23 and 6.1 Å from the C5 position of the cytosine residue (Table 1).

The 5-halogenated pyrimidines have been previously used in physical studies of DNA-protein interactions, and in particular, it has been shown that a 5-bromo substituent can substitute well for a methyl group. Emerging evidence, however, suggests that 5-chlorocytosine and 5-bromocytosine must also be included as endogenous DNA damage products. Myeloperoxidase generates HOCl, and eosinophil peroxidase generates HOBr (14–17). Both of these reactive halogenating species are essential components of innate immunity in mammals, and defects in the generation of either are associated with increased susceptibility to infection (27). As with other reactive oxygen species, such as hydrogen peroxide, the reactive halogenating species can also cause collateral damage in normal cells. It is estimated that endogenous levels of HOCl generated from myeloperoxidase-mediated reactions range from 20 to 400 μmol/L (28–32). It has been shown that, within these physiologic levels of HOCl, the main lesion produced in DNA is 5-chlorocytosine and that 5-chlorocytosine is detectable in human leukocytes even in the absence of treatment with HOCl (17). Furthermore, there is evidence that chlorination damage may be more long lived than bromination damage due to the absence of detectable repair activity against 5-chlorocytosine (11, 18). The effect of halogenation damage products associated with inflammation on epigenetics is only now becoming apparent. Further studies *in vivo* systems will determine the extent by which these endogenous DNA damage products contribute to the changes in methylation patterns seen in human tumors.

The results of the studies reported here indicate that the heritable transmission of cytosine methylation patterns following cell replication could be altered by endogenous DNA damage: oxidation tends to interfere with methylation, whereas 5-chlorocytosine and 5-bromocytosine can mimic 5-methylcytosine, resulting in fraudulent methylation of previously unmethylated sites (Fig. 1*C*). In a similar manner, oxidation damage tends to interfere with the binding of MBPs, whereas 5-chlorocytosine and 5-bromocytosine mimic 5-methylcytosine in facilitating binding. Although the precise mechanisms by which methylation patterns are established in cells are as yet unknown, endogenous DNA damage is likely to interfere with the faithful transmission of methylation patterns following cell replication and may account in part for epigenetic perturbations observed in human tumors.

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